Genetic engineering of proteins with cell membrane permeability

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Received 14 October 1997; accepted 24 February 1998

The discovery of methods for generating proteins with inherent cell membrane-translocating activity will expand our ability to study and manipulate various intracellular processes in living systems. We report a method to engineer proteins with cell-membrane permeability. After a 12–amino acid residue membrane-translocating sequence (MTS) was fused to the C-terminus of glutathione S-transferase (GST), the resultant GST-MTS fusion proteins were efficiently imported into NIH 3T3 fibroblasts and other cells. To explore the applicability of this nondestructive import method to the study of intracellular processes, a 41-kDa GST-Grb2SH2-MTS fusion protein containing the Grb2 SH2 domain was tested for its effect on the epidermal growth factor (EGF)-stimulated signaling pathway. This fusion protein entered cells, formed a complex with phosphorylated EGF receptor (EGFR), and inhibited EGF-induced EGFR–Grb2 association and mitogen-activated protein kinase activation.

Keywords: drug delivery, epidermal growth factor, protein delivery, SH2 domain

The concentration of specific proteins within cells can be significantly enhanced by gene transfection with appropriate constructs, by microinjection of individual cells, or by various other delivery methods. These techniques have been shown to be useful for studying and influencing intracellular processes in living systems. The hydrophobic region (h region) of a signal peptide sequence has been used as a carrier to deliver various short peptides (the cargo) into living cells in a nondestructive manner[1]. This method of peptide import has been applied to studies of various intracellular protein functions and cellular processes, such as the nuclear translocation of transcription factor-kappa B[1], intracellular functions of fibroblast growth factor-1[2], the Ras activation cascade originating from the epidermal growth factor (EGF) receptor (EGFR)[3], intracellular integrin signaling[4], and functional roles of Shc adaptor protein[4].

In cell-permeable peptide design, the short h region of a signal peptide, used as a carrier for import, can be placed at either the N-terminus[1-3] or the C-terminus of the cargo peptide[4]. Both orientations mediate similar levels of peptide cellular import. A 25–amino acid residue peptide is the largest cargo thus far delivered into cells using the signal peptide-based membrane-translocating sequence (MTS)[1]. To determine if a similar MTS could mediate the cellular import of an entire protein, we genetically engineered *Schistosoma japonicum* glutathione *S*-transferase (GST; EC 2.5.1.18) to contain a 12-residue MTS at its C-terminus. We show that the GST-MTS fusion protein, expressed in *Escherichia coli* and purified by glutathione-agarose affinity chromatography, was efficiently imported into living cells. When the Grb2 SH2 domain[5] was fused to GST-MTS, the resulting 41-kDa GST-Grb2SH2-MTS protein was imported into cells and inhibited EGF-induced EGFR–Grb2 association and MAP kinase activation.

Results

Design, expression, and purification of GST containing a membrane-translocating sequence. To determine whether a peptide-based MTS can be used to mediate the delivery of an entire protein into living cells, we genetically engineered GST to contain a 12-residue MTS at its C-terminus (referred to as GST-MTS). The MTS used was modified from the 16-residue h region of the signal sequence of Kaposi fibroblast growth factor[4], which has been used as the carrier for many cell-permeable peptides[1-3,4] (Fig. 1A). GST was selected for use as a model protein for these studies because it can be expressed to high levels in *E. coli* and is easily purified to near homogeneity in a single affinity chromatography step. For the construction of plasmids expressing GST-MTS fusion proteins, complementary oligonucleotides were synthesized according to the designed MTS sequence. Two different GST-MTS expression plasmids, pGEX-3X-MTS1 and pGEX-3X-MTS2, were constructed. The location of BamHI sites in these two plasmids was different so that a potential target protein or protein domain could be expressed with the MTS as either an N-terminal or a C-terminal extension (Fig. 1A). Both plasmids contained a factor X cleavage site between GST and the MTS, which allowed us to characterize the attached MTS following enzyme cleavage. Wild-type GST (GST-WT), GST-MTS1, and GST-MTS2 were expressed in *E. coli* strain DH5α and purified from bacterial cell lysates by glutathione-agarose affinity chromatography. Analysis of the purified GST-MTS1 and GST-MTS2 protein preparations by SDS-PAGE showed predominantly single protein bands (29 kDa) with the predicted increase in apparent molecular size relative to GST-WT (Fig. 1B). To confirm the amino acid content of the MTS in GST-MTS proteins, we cleaved the GST- MTS1 protein with factor Xa and purified the MTS-containing peptide by high performance liquid chromatography (HPLC). The molecular mass of the purified MTS-containing peptide was determined by mass spectrometry analysis and was found to match exactly the predicted value (mass, calculated: 1931 Da; observed: 1931 Da).

Cellular import of the GST protein with a C-terminal MTS. Having produced the GST-MTS proteins in a highly purified form, we next questioned whether these proteins were capable of penetrating the cell membrane of living cells. To test this possibility, we used GST-MTS1 and NIH 3T3 cells for protein import studies